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Precursor lesions of vulvar squamous cell carcinoma – histology and biomarkers: A systematic review

Shatavisha Dasgupta[a,*], Patricia C. Ewing-Graham[a], Sigrid M.A. Swagemakers[a,b], Peter J. van der Spek[a,b], Helena C. van Doorn[c], Vincent Noordhoek Hegt[a], Senada Koljenović[a], Folkert J. van Kemenade[a]

[a] Department of Pathology, Erasmus MC, University Medical Centre Rotterdam, the Netherlands
[b] Department of Bioinformatics, Erasmus MC, University Medical Centre Rotterdam, the Netherlands
[c] Department of Gynecologic Oncology, Erasmus MC Cancer Institute, Rotterdam, the Netherlands

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ABSTRACT

The precursor lesion of vulvar squamous cell carcinoma (VSCC), namely vulvar intraepithelial neoplasia (VIN), is classified as: human papillomavirus (HPV)-related high grade squamous intraepithelial lesion (HSIL), and HPV-independent differentiated VIN (dVIN). Traditionally, histology and immunohistochemistry (IHC) have been the basis of diagnosis and classification of VIN. HSIL shows conspicuous histological atypia, and positivity on p16-IHC, whereas dVIN shows less obvious histological atypia, and overexpression or null-pattern on p53-IHC. For both types of VIN, other diagnostic immunohistochemical markers have also been evaluated. Molecular characterization of VIN has been attempted in few recent studies, and novel genotypic subtypes of HPV-independent VSCC and VIN have been identified.

This systematic review appraises the VSCC precursors identified so far, focusing on histology and biomarkers (immunohistochemical and molecular). To gain further insights into the carcinogenesis and to identify additional potential biomarkers, gene expression omnibus (GEO) datasets on VSCC were analyzed; the results are presented.

1. Introduction

Vulvar squamous cell carcinoma (VSCC) constitutes 90% of all vulvar malignancies, and arises from the precursor lesion, vulvar intraepithelial neoplasia (VIN) (WHO, 2014). Around 1/3rd of VSCC are caused by human papillomavirus (HPV), and the precursor lesion for this group is usual VIN/high grade squamous intraepithelial lesion (uVIN/HSIL) (WHO, 2014; Bornstein, 2016). The majority of VSCC are, however, HPV-independent and arise on the background of chronic dermatoses. Somatic mutations of *TP53* have been implicated in the pathogenesis of this category, and the precursor lesion is called differentiated VIN (dVIN) (WHO, 2014; Bornstein, 2016).

Traditionally, histology and immunohistochemistry (IHC) have been the basis of diagnosis and classification of VIN and VSCC. In recent years, for several cancers, advanced molecular analyses have allowed more objective classification, and identification of diagnostic and prognostic biomarkers. For VIN and VSCC, molecular characterization has been attempted in a limited number of studies so far.

This systematic review was performed with the objective of appraising the histological features of, and the biomarkers (immunohistochemical and molecular) for VSCC precursors. To gain further insights into VSCC carcinogenesis, publicly available datasets on expression profiling of VSCC were analyzed; these results are also presented.

2. Materials and methods

2.1. Literature review

The recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) protocol were followed (Anon, 2019a). The review was registered in the International

* Corresponding author at: Department of Pathology, Erasmus MC, University Medical Centre Rotterdam, Postbus 2040, Be-building, 3000CA, Rotterdam, the Netherlands.
E-mail addresses: s.dasgupta@erasmusmc.nl (S. Dasgupta), p.ewing@erasmusmc.nl (P.C. Ewing-Graham), s.swagemakers@erasmusmc.nl (S.M.A. Swagemakers), p.vanderspek@erasmusmc.nl (P.J. van der Spek), h.vandoorn@erasmusmc.nl (H.C. van Doorn), hegt123@gmail.com (V. Noordhoek Hegt), s.koljenovic@erasmusmc.nl (S. Koljenović), f.vankemenade@erasmusmc.nl (F.J. van Kemenade).

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Prospective Register of Systematic Reviews (PROSPERO), with accession number CRD42019107290 (Anon, 2019b).

Electronic search strategies combining Medical Subject Headings (MeSH) and free-text words were prepared, with the help of medical librarians at Erasmus MC. Biomedical bibliographic databases, viz. Embase.com, MEDLINE Epub (Ovid), Cochrane Central Register for Controlled Trials (CENTRAL), Web of Science (Science and Social Science Citation Index), were searched. The full search strategy is provided in supplementary document 1. Date restriction was not applied. The last search was conducted in May 2019. Citation, reference, and hand searching were additionally performed.

Original articles and review articles describing either the histology, or immunohistochemical or molecular markers of VSCC precursors, and written in English were included. Case reports, conference abstracts, animal studies, and in-vitro studies were excluded.

A total of 1112 references were retrieved, of which 373 were included after the first round of screening by one author (SDG). These 373 references were independently screened by three other authors (PEG, SK, and FvK) by reading the titles. PEG, SK, and FvK are practising pathologists with substantial experience in the subject content. One hundred and fifty four references met consensus for inclusion; full text was available for 127 of these. Nine additional references were identified through reference searches. The final inclusion constituted 106 articles (99 original articles, 7 reviews). The process of reference selection is delineated in Fig. 1.

Due to variability in the nomenclature, and heterogeneity in the published data, a meta-analysis could not be performed, and a narrative synthesis was prepared.

2.2. Gene expression omnibus (GEO) DataSet analysis

Datasets on gene expression analyses of VSCC were searched on GEO. Four datasets were identified, of which 3 were discarded; 2 owing to very low number of samples, and 1 due to lack of intensity values. The included dataset (GSE38228) contained gene expression data from 14 VSCC and 5 normal vulvar samples, performed on Illumina Human HT-12 V4.0 (Micci et al., 2013). The signal intensity data had been ^{2}log transformed (base2) and quantile normalised (Bolstad et al., 2003).

The data was imported into OmniViz (version 6.1.13.0, BioWisdom Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensity of all samples was calculated, and the level of expression was assessed relative to this geometric mean and ^{2}log-transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes between VSCC and controls were identified using statistical analysis of microarrays (SAM). Cutoff values for significantly expressed genes were a false discovery rate (FDR) of $\leq$ 0.01, and a fold change of 1.5. Functional annotation of the SAM results was done using Ingenuity Pathway Analysis (IPA, QIAGEN, Mountain View, CA), and Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8, NIAID/NIH).

3. Results

The information extracted from the literature, and the results of GEO analyses were categorised as pertaining to HPV-related VSCC precursors, or HPV-independent VSCC precursors, and are presented in the subsequent sections. The evolution of terminology, etiopathogenesis, and clinical features for both categories, extracted from the included literature, are also briefly discussed.

3.1. Evolution of terminology

The term intraepithelial neoplasia was introduced in 1967, and was adopted by the International Society for the Study of VulvoVaginal Diseases (ISSVD) in 1986 (Crum et al., 1982; Wilkinson et al., 1986). Initially, a three-tier grading system for VIN (VIN I, II, III) was recommended. The VINI category was later removed, as this almost never progressed to VSCC (Sideri et al., 2005). In 2004, the ISSVD scheme was modified to include usual VIN (uVIN), and differentiated VIN (dVIN), to signify HPV-related and HPV-independent VSCC precursors respectively (Sideri et al., 2005).

With a view to achieving uniformity in terminology, in 2012, the Lower Anogenital Squamous Terminology (LAST) committee recommended the terminology of squamous intraepithelial lesion (SIL) for VSCC precursors, classified as low grade SIL (LSIL) and high grade SIL (HSIL) (Darragh et al., 2012). The 2014 World Health Organization (WHO), and 2015 ISSVD classifications accepted the SIL terminology for HPV-related VIN, and retained dVIN as a separate category (WHO, 2014; Bornstein, 2016). In this review, we used HSIL, and dVIN to refer to the precursors of HPV-related VSCC and HPV-independent VSCC respectively.

3.2. HPV-related VSCC precursors (HSIL)

3.2.1. Etiopathogenesis

More than 80% of HSIL have been reported to be HPV-positive, frequently detected genotypes being high-risk HPV16 (77.2%), HPV33 (10.6%), and HPV18 (2.6%) (van de Nieuwenhof et al., 2008; Scurry et al., 2006; Medeiros et al., 2005; van Beurden et al., 1995; Bonvicini et al., 2005; Bornstein et al., 1988; Buscema et al., 1988; Haefner et al., 1995; Park et al., 1991).

Similar high rates of HPV-positivity, however, have not been found in VSCC. Only 28.6% of cases from an international cohort of 1709 VSCC were found to harbor HPV, in 75% of which, HPV16 was detected (de Sanjosé et al., 1990). Other studies have reported 15–79% HPV-positivity in VSCC (del Pino et al., 2013). Low-risk HPV6 and HPV11 have been infrequently reported in VSCC; their exact role in the carcinogenesis is unclear (Iwasawa et al., 1997; Hampl et al., 2007).

High-risk HPV mediates carcinogenesis primarily through the oncoproteins E6 and E7; these interfere with the functioning of tumor suppressor retinoblastoma protein (pRB), and p53 (Lerma et al., 2002). Loss of pRB generates oncogenic stress, potentially leading to $p16^{INK4a}$ over-expression, and thus allowing hyper-proliferation of the infected cells (Lerma et al., 2002; Riethdorf et al., 2004).

To facilitate carcinogenesis, persistent HPV-infection also induces a local immunosuppressive microenvironment, characterized by reduced concentrations of (CD1a+ and CD207+) dendritic cells and HPV-specific (CD4+ and CD8+) T-cells, and upregulation of T-regulatory cells (Santegoets et al., 2008; Preti et al., 2014; Terlou et al., 2010). Treatment with imiquimod is known to normalize (CD4+, CD8+) T-cell counts in the epidermis, and bring about viral clearance (Terlou et al., 2010).

3.2.2. Clinical features

HSIL most commonly affects women of 40–44 years, with a second peak after 55 years (van Beurden et al., 1995; de Sanjosé et al., 1990; Terlou et al., 2010; Jones et al., 2005). Patients commonly present with pain, pruritus, or dysuria, although 40% can be asymptomatic (Al-Ghamdi et al., 2002). Clinically, these lesions can be whitish, erythematous, or pigmented macules, papules, or verrucous plaques (van Beurden et al., 1995; McNally et al., 2002; Chafe et al., 1988; van Seters et al., 2005). HSIL can be multifocal in 70% of cases, and the commonest locations are the labia minora and perineum (Al-Ghamdi et al., 2002; Chafe et al., 1988; van Seters et al., 2005). Due to the 'field effect' induced by HPV, patients with HSIL may have concurrent involvement of other ano-genital sites (Preti et al., 2014; Jones et al., 2005; McNally et al., 2002; Chafe et al., 1988; van Seters et al., 2005; Léonard et al., 2014; van der Avoort et al., 2006). A thorough examination of the entire lower genital tract is therefore recommended for all HSIL patients.

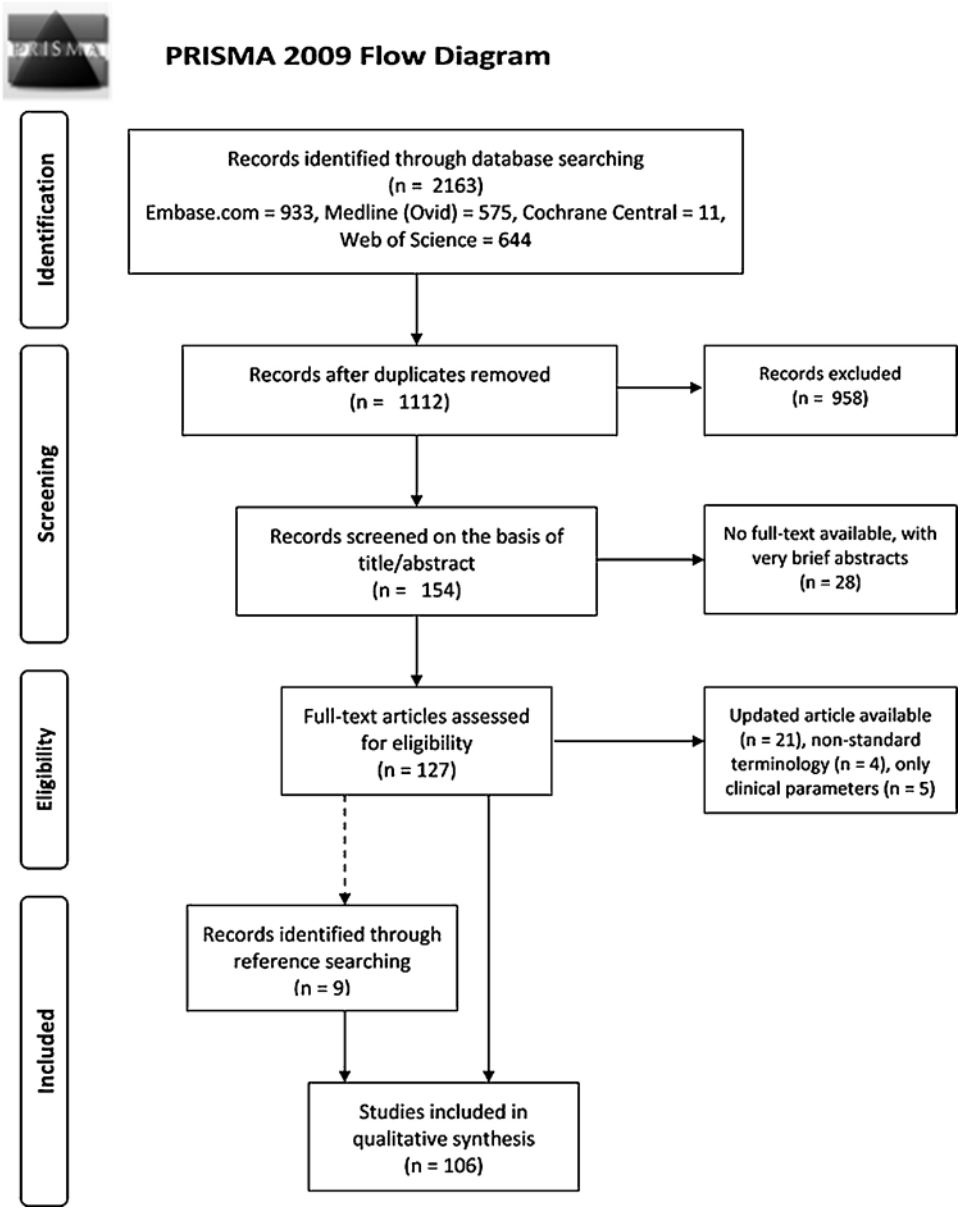

Fig. 1. PRISMA flow diagram depicting the process of reference selection.

HSIL has a relatively low risk of progression to VSCC: 9–16% for untreated cases, and 3% for treated cases. Around 1.2% of HSIL spontaneously regress (Jones et al., 2005; McNally et al., 2002; Léonard et al., 2014; Sykes et al., 2002). The reported recurrence rate for HSIL varies between 13–36% (Preti et al., 2014; McNally et al., 2002; Sykes et al., 2002; Preti et al., 2015).

3.2.3. *Histological features*

Histological features of HSIL were extracted from 9 studies (Preti et al., 2014; Knight, 1943; Baggish et al., 1989; Shatz et al., 1989; Hart, 2001; Scurry and Wilkinson, 2006; Hoang et al., 2016; Cohen et al., 2019; Rakislova et al., 2018). The architectural and cytological abnormalities of HSIL are conspicuous, and often apparent under low magnification [Fig. 2]. A basophilic (basaloid) appearance can frequently be appreciated in HSIL (Cohen et al., 2019). The key identifying features include high nuclear-to-cytoplasmic ratio, hyperchromasia, pleomorphism, multinucleated cells, mitoses, and apoptotic bodies [Fig. 2] (Hoang et al., 2016). The disturbed orientation of the dysplastic epithelial cells often render a 'wind-blown' appearance to HSIL (Hoang et al., 2016). Tangential sectioning of HSIL with extensive downgrowths, or with involvement of skin appendages, can mimic early invasion and these should be interpreted with caution (Baggish et al., 1989; Shatz et al., 1989; Hart, 2001). The histological features are listed in Table 1.

Two histological variants of HSIL have been reported: warty/condylomatous, and basaloid/undifferentiated (Hoang et al., 2016). Warty HSIL has an acanthotic or papillary surface, with deep and wide rete ridges, abundant koilocytes, and dyskeratotic cells (Scurry and Wilkinson, 2006; Hoang et al., 2016). Basaloid HSIL is flat, with small, uniform, basaloid cells often replacing the entire epithelium (Scurry and Wilkinson, 2006; Hoang et al., 2016). Since some HSIL exhibit histological features from both variants, it has been assumed that these are a part of the same spectrum.

HSIL can also histologically mimic dVIN, with a pooled prevalence of 2% (Cohen et al., 2019; Rakislova et al., 2018; Faber et al., 2017). Similarly to dVIN, these show cytoplasmic eosinophilia due to abnormal keratinization, spongiosis, and atypia limited to the basal and parabasal layers (Rakislova et al., 2018). Rakislova et al. demonstrated p16-positivity, and detected HPV16 in these dVIN-like HSIL (Rakislova et al., 2018). HSIL with superimposed lichen simplex chronicus (LSC)

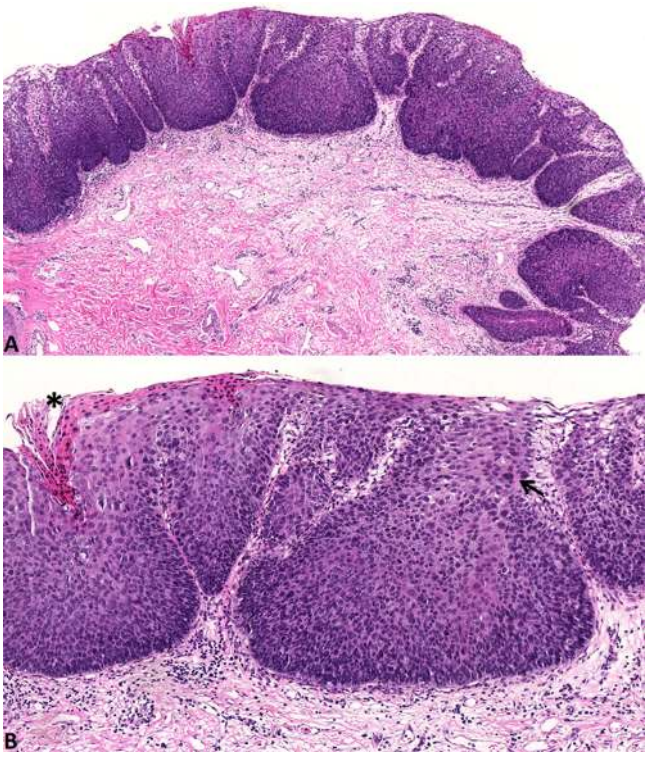

Fig. 2. High grade squamous intraepithelial lesion (HSIL) with characteristic histological features; hematoxylin-eosin (HE) stain. A. Acanthosis with club shaped rete ridges, and a basophilic appearance of the epithelium can be appreciated under low magnification (original magnification 25X). B. Cellular crowding, anisonucleosis, pleomorphic cells with high nuclear cytoplasmic ratio (arrow), hyperkeratosis and parakeratosis (asterisk) can be observed under higher magnification (original magnification 100X).

can also mimic dVIN histologically, but can be correctly diagnosed through p16-positivity on IHC (Watkins et al., 2019).

3.2.4. Immunohistochemical markers

Immunohistochemical markers studied in HSIL are presented below, and summarized in Table 2. This information was extracted from 24 studies (Lerma et al., 2002; Riethdorf et al., 2004; van der Avoort et al., 2006; Rakislova et al., 2018; Watkins et al., 2019; Yang and Hart, 2020; Dong et al., 2015; Cheng et al., 2015; Hoevenaars et al., 2008; Jeffreys et al., 2018; Rolfe et al., 2001; Samartzis et al., 2011; Li et al., 2013; Brustmann and Brunner, 2013; Brustmann et al., 2011; Goyal et al., 2018; Chen et al., 2010; Wellenhofer and Brustmann, 2012; Pinto et al., 2013; Nooij et al., 2016; Stewart and Crook, 2014; Podoll et al., 2016; Ekeowa-Anderson et al., 2012; Bovo et al., 2004). The immunohistochemical markers have been categorized as per their subcellular location as nuclear, cytoplasmic, or extra-cellular. The biological processes and canonical pathways associated with these markers are presented in Table S1(supplementary document 2).

3.2.4.1. Nuclear markers

(i) p16: As per LAST guidelines, continuous/diffuse/band-like/block-like strong nuclear, or nuclear with cytoplasmic p16-expression in the basal layer, extending up to at least one-third of the epithelial thickness, is to be interpreted as 'positive' p16-staining [Fig. 3] (Darragh et al., 2012). Cytoplasmic staining alone, or patchy focal staining without staining in the basal layers should be interpreted as p16-negative.

Positivity with p16-IHC has been reported for stand-alone HSIL, as well as HSIL adjacent to VSCC (Lerma et al., 2002; Rakislova et al., 2018; Watkins et al., 2019; Yang and Hart, 2020; Dong et al., 2015; Cheng et al., 2015; Hoevenaars et al., 2008; Jeffreys et al., 2018). With p16-IHC, improvement in the inter-observer agreement in HSIL

Table 1
Histological features of HSIL, and dVIN

Histological features of HSIL		
Knight (1943)	*Other literature* (Preti et al., 2014; Knight, 2020; Baggish et al., 1989; Shatz et al., 1989; Hart, 2001; Scurry and Wilkinson, 2006; Hoang et al., 2016; Cohen et al., 2019; Rakislova et al., 2018)	*WHO 2014 criteria* (WHO, 2014)
1 Hyperkeratosis	1 High nuclear-cytoplasmic ratio	1
2 Parakeratosis	2 Hyperchromasia	1
3 Acanthosis with club shaped rete ridges	3 Pleomorphism	1
4 Disorientation of the individual cells commencing above the basal cell layer with variable extension to the surface	4 Multinucleated cells	1 Epithelial cell hyperchromasia
5 Nuclear clumping with mitotic figures	5 Mitoses	2 Cellular crowding
6 Intact basement membrane	6 Apoptotic bodies	3 Anisonucleosis
		4 Acanthosis
		5 Parakeratosis
		6 Hyperkeratosis
		7 Variable HPV cytopathic effect
Histological features of dVIN		
Yang and Hart (Yang and Hart (2020))	*Other literature* (Preti et al., 2014; Hart, 2001; Hoang et al., 2016; Cohen et al., 2019; Bigby et al., 2016; Reutter et al., 2016; van den Einden et al., 2013; Dasgupta et al., 2018; Singh et al., 2015; Ordi et al., 2009)	*WHO 2014 criteria* (WHO, 2014)
1 Variable nuclear atypia, from slight-to-moderate degree, to occasionally severe	1 Basal cellular atypia (including disarray of the basal cellular layers, large pleomorphic keratinocytes)	1 Basal cell atypia, with nuclear hyperchromasia
2 Hyperchromatic nuclei with irregular contours in the basilar cells	2 Angulated nuclei	2 Karyomegaly
3 Vesicular nuclei	3 Atypical mitosis in the basal layer	3 Prominent nucleoli
4 Macronucleoli	4 Mitotic count > 5 / 5mm	4 Atypical mitoses in the basal layer
5 Binucleated cells	5 Prominent nucleoli/Macronucleoli	5 Dyskeratosis
6 Scattered mitoses, in basilar and suprabasilar layers	6 Individual cell keratinisation	6 Individual cell keratosis
7 Enlarged cells	7 Deep keratinisation	7 Elongation and anastomosis of rete ridges
8 Elongated and frequently branched rete ridges	8 Deep squamous eddies	
9 Abundant, brightly eosinophilic cytoplasm	9 Cobblestone appearance	
10 Prominent intercellular bridges	10 Hypermaturation of rete ridges	
11 Whorls of differentiated cells, with/without keratin pearls	11 Elongation and anastomosis of rete ridges	
	12 Hyperplasia/Acanthosis	
	13 Hyperkeratosis	
	14 Parakeratosis	

Table 2
Immunohistochemical markers that have been studied for HSIL, and dVIN.

Biomarker	Lesion(s) studied	Location	Molecular Functions*
p16 (CDKN2A) (Darragh et al., 2012; Lerma et al., 2002; Riethdorf et al., 2004; Rakislova et al., 2018; Faber et al., 2017; Watkins et al., 2019; Yang and Hart, 2020; Dong et al., 2015; Cheng et al., 2015; Hoevenaars et al., 2008; Jeffreys et al., 2018)	HSIL, dVIN	nucleus	transcription regulator; DNA binding; NF-κß binding; p53 binding; protein kinase binding; RNA binding; ubiquitin-protein ligase inhibitor activity
p53 (Yang and Hart, 2020; Jeffreys et al., 2018; Wellenhofer and Brustmann, 2012; Santos et al., 2004; Hantschmann et al., 2005; Liegl and Regauer, 2006; Rolfe et al., 2003; Vanin et al., 2002)	HSIL, dVIN	nucleus	transcription regulator; DNA binding; histone deacetylase regulator activity; protein heterodimerization/homodimerization activity
CCND1 (Lerma et al., 2002; Rolfe et al., 2001)	HSIL, dVIN	nucleus	transcription regulator; histone deacetylase binding; kinase activity
pRB (Lerma et al., 2002; Rolfe et al., 2001)	HSIL	nucleus	sequence-specific DNA binding; transcription factor; ubiquitin protein ligase binding
Ki-67/MIB-1 (van der Avoort et al., 2006; Hoevenaars et al., 2008)	HSIL, dVIN	nucleus	ATP binding; nucleotide binding; protein binding
HDAC1 (Samartzis et al., 2011)	HSIL	nucleus	transcription regulator; NF-kappaB binding; p53 binding
CTNNB1 (Li et al., 2013)	HSIL, dVIN	nucleus	transcription regulator; cadherin binding; chromatin binding; SMAD binding
CDH1 (Li et al., 2013)	HSIL**	plasma membrane	alpha-catenin binding; beta-catenin binding; cell adhesion molecule binding; cytoskeletal protein binding; gamma-catenin binding
SOX2 (Brustmann and Brunner, 2013)	HSIL, dVIN	nucleus	transcription regulator
γ-H2AX (Brustmann et al., 2011)	HSIL	nucleus	transcription regulator
Survivin (Brustmann et al., 2011; Wellenhofer and Brustmann, 2012)	HSIL	cytoplasm	chaperone binding; cofactor binding; cysteine-type endopeptidase inhibitor activity involved in apoptotic process; enzyme binding; protein heterodimerization / homodimerization activity; Ran GTPase binding; tubulin binding
GATA3 (Goyal et al., 2018)	HSIL, dVIN	nucleus	transcription regulator; HMG box domain binding; interleukin-2 receptor binding
MCM2 (Chen et al., 2010)	HSIL	nucleus	enzyme - 3′-5′ DNA helicase activity; ATP binding; histone binding
TOP2A (Chen et al., 2010)	HSIL	nucleus	enzyme - ATPase activity; histone deacetylase binding; ubiquitin binding
hTERT (Wellenhofer and Brustmann, 2012)	HSIL	nucleus	enzyme - nucleotidyltransferase activity; protein binding; tRNA binding
p-S6 (Pinto et al., 2013)	HSIL, dVIN	nucleus	mRNA binding; protein binding; structural constituent of ribosome
STMN1 (Nooij et al., 2016)	HSIL	cytoplasm	protein binding; tubulin binding
FSCN1 (Stewart and Crook, 2014)	HSIL, dVIN	cytoplasm	actin binding; cadherin binding; RNA binding
CK17 (Podoll et al., 2016; Dasgupta et al., 2018)	HSIL, dVIN	cytoplasm	MHC class II protein binding / receptor activity; structural constituent of cytoskeleton
AKT1 (Ekeowa-Anderson et al., 2012)	HSIL	cytoplasm	enzyme - kinase activity, transferase activity; calmodulin binding
MMP2 (Bovo et al., 2004)	HSIL	extracellular space	enzyme - hydrolase activity, metalloendopeptidase activity
CK13 (Dasgupta et al., 2018)	dVIN	cytoplasm	protein binding; structural molecule activity
ER (Zannoni et al., 2011)	dVIN	nucleus	ligand-dependent receptor; DNA binding; transcription factor binding
PR (Zannoni et al., 2011)	dVIN	nucleus	ligand-dependent receptor; DNA binding

* From IPA Gene View (QIAGEN).
** This marker was studied in dVIN, but the pattern of expression was same as that in the normal vulvar epithelium.

diagnosis has been reported (Darragh et al., 2012).

The combination of positive HPV-polymerase chain reaction (HPV-PCR), and block-positive p16-IHC is considered to be the 'gold standard' for diagnosing HSIL (Cohen et al., 2019). However, p16-IHC alone is considered a reliable surrogate marker for HPV-infection (Riethdorf et al., 2004; Yang and Hart, 2020; Dong et al., 2015; Cheng et al., 2015; Hoevenaars et al., 2008). p16-IHC can also help distinguish HSIL from benign mimics, such as transition zone mucosa, squamous hyperplasia, or radiation change, which are p16-negative (Darragh et al., 2012).

Fig. 3. High grade squamous intraepithelial lesion (HSIL), histology and p16 and p53 immunohistochemistry (IHC), original magnification 50X. A. Hematoxylin-Eosin (HE) stained section shows the characteristic histology of HSIL. B. Block-like positivity can be appreciated on p16-IHC. C. On p53-IHC, scattered nuclear staining in the suprabasal layers, with lack of staining in the basal layers is seen.

(i) p53: On p53-IHC, HSIL usually shows wild-type (wt) staining, characterized by sporadic nuclear staining, with weakly positive to completely negative basal epithelial layer [Fig. 3]. In some HSIL, 'accentuated wt-p53' pattern is seen, characterized by weak, patchy staining in the basal layer, and a higher proportion of positive nuclei in the suprabasal layers (Jeffreys et al., 2018).

(ii) Cyclin-D1 and pRB: Loss of pRB-expression in HSIL and VSCC reflects disruption of pRB by HPV. The cyclinD1-CDK4 complex reverses the tumor suppressor effect of pRB, and allows cell-cycle progression. Normal vulvar epithelium is negative for cyclin-D1, whereas its over-expression in both the nucleus and cytoplasm has been reported in both HSIL and VSCC (Lerma et al., 2002; Rolfe et al., 2001).

(iii) Histone deacetylases-class I (HDAC1): HDACs represses transcription by increasing the affinity of histone complexes for DNA, and also modifies the transcription factors p53, E2F, and pRB. Increased nuclear expression of HDAC1,2, and 3 have been reported for HSIL and VSCC (Samartzis et al., 2011).

(iv) E-Cadherin (CDH1)/ß-Catenin (CTNNB1): E-cadherin/ß-catenin complex regulates cellular adhesion, proliferation, and survival. Dysfunction of this complex has been implicated in carcinogenesis (Li et al., 2013). In non-neoplastic vulvar lesions, CDH1 and ß-catenin are strongly expressed in the epithelial cell membranes (Li et al., 2013). In HSIL, expression of both in the cytoplasm, with or without expression in the cell membrane is seen (Li et al., 2013).

(v) SRY-box 2 (SOX2): SOX2 controls pluripotency in both embryonic and adult tissue-specific stem cells, and can induce pluripotency in adult somatic cells (Brustmann and Brunner, 2013). Weak nuclear SOX2-expression in the basal and parabasal epithelial layers is seen in normal vulva. Increased, strong nuclear SOX2-expression, particularly in the middle and upper-third of the epithelium, has been reported for HSIL (Brustmann and Brunner, 2013).

(vi) H2A histone family member X (γ-H2AX): γ-H2AX is generated from phosphorylation of the histone protein H2AX, in response to DNA-double strand breaks. γ-H2AX induces chromatin modification, and apoptosis (Brustmann et al., 2011). Diffuse or granular nuclear staining with γ-H2AX in all the epithelial layers has been reported for HSIL (Brustmann et al., 2011).

(vii) GATA-binding protein 3 (GATA3): GATA3 is known to upregulate the MDM2-proto-oncogene, which in turn downregulates and degrades p53. Moderate to strong nuclear GATA3-expression in non-neoplastic vulva, as well as in HSIL has been reported (Goyal et al., 2018).

(viii) ProEx C: ProEx C is an antibody cocktail targeting the nuclear enzymes minichromosome maintenance complex component 2 (MCM2), and topoisomerase IIa (TOP2A). MCM2 influences mitotic G1/S phase transition. TOP2A modulates chromosome condensation and segregation, and is activated in response to genotoxic stress. For HSIL, diffuse nuclear staining with ProEx C, from lower one-third to full epithelial thickness, has been reported. In normal vulvar epithelium, ProEx C only stains the basal and parabasal layers (Chen et al., 2010).

(ix) Human Telomerase Reverse Transcriptase (hTERT): hTERT extends and maintains telomeres, and enables cells to overcome replicative senescence. In HSIL, nuclear staining with hTERT follows the distribution of atypical keratinocytes, being limited to < 50% of the epithelial cells in most cases. hTERT staining only in the basal/parabasal layers has been noted in normal vulvar epithelium (Wellenhofer and Brustmann, 2012).

(x) Ki-67/MIB-1: Ki-67 is a nuclear antigen present in proliferating human cells in all stages of the cell-cycle, except the G0 phase (van der Avoort et al., 2006; Hoevenaars et al., 2008). MIB-1 is the monoclonal antibody against Ki-67. In normal vulvar epithelium, MIB-1 stains mainly the parabasal layers, and infrequently the basal layers (Hoevenaars et al., 2008). In HSIL, increased MIB-1 staining in both basal and parabasal layers, extending into the upper two-thirds of the epithelium can be seen (van der Avoort et al., 2006; Hoevenaars et al., 2008).

(xi) Phosphorylated-S6 (p-S6): Hyperactivity of the EGFR/ERK, and PI3K/AKT/mTOR signaling pathways, which are involved in SCC, leads to phosphorylation of the ribosomal protein S6, which modulates mRNA translation (Pinto et al., 2013). In normal vulvar epithelium, the basal and suprabasal cells are negative for p-S6, whereas in HSIL, the basal cells show nuclear p-S6-expression (Pinto et al., 2013).

3.2.4.2. Cytoplasmic markers

(i) Stathmin (STMN1): STMN1 is a microtubule-destabilising phosphoprotein, which regulates mitosis (Nooij et al., 2016). Normal vulvar mucosa does not express STMN1, whereas in HSIL, cytoplasmic expression in more than one-third of the epithelial thickness has been reported (Nooij et al., 2016).

(ii) Fascin-1 (FSCN1): FSCN1, an actin-bundling protein, promotes migration and invasion of carcinoma cells. Increased cytoplasmic FSCN1-expression in all epithelial layers, except in the surface parakeratotic cells, has been reported for HSIL (Stewart and Crook, 2014). FSCN1 immunoreactivity is limited to the lower-third of the epithelium in normal vulva.

(iii) Cytokeratin 17 (CK17): The intermediate filament protein CK17, regulates protein synthesis and cell growth, and is expressed in activated keratinocytes in the suprabasal layers of epidermis. In HSIL, focal cytoplasmic CK17-staining of weak or moderate intensity, in the superficial epithelial layers, has been reported (Podoll et al., 2016).

(iv) Survivin: Apoptosis-inhibitor protein survivin is overexpressed in human cancers, and is considered an unfavorable prognostic marker (Brustmann et al., 2011; Wellenhofer and Brustmann, 2012). In normal vulva, survivin stains the cytoplasm of parabasal/basal epithelial cells, whereas in HSIL, the staining pattern has been reported to follow the extension of the atypical cells within the epithelium (Wellenhofer and Brustmann, 2012).

(v) AKT1: AKT1 is a serine/threonine kinase, down-regulated by cutaneous HPV-types to weaken the keratin envelope and allow viral release (Ekeowa-Anderson et al., 2012). AKT1 loss in HSIL correlates with high copy numbers of episomal HPV16, i.e. is indicative of early HPV-infection. Expression of AKT1 in HSIL correlates with low copy numbers of episomal HPV16, and indicates HPV-integration (Ekeowa-Anderson et al., 2012).

3.2.4.3. Extracellular markers. Matrix metalloproteinase (MMP2): MMP2, an enzyme of the metalloproteinase family, degrades type IV collagen and fibronectin in the basement membrane, and facilitates stromal and vascular invasion by tumor cells (Bovo et al., 2004). Granular or diffuse cytoplasmic staining with MMP-2 in stromal cells has been reported in HSIL and VSCC (Bovo et al., 2004).

3.2.5. Molecular markers

3.2.5.1. Allelic imbalances, loss of heterozygosity (LOH), copy number alterations (CNA). Loss of chromosome (chr) 3p and gain of chr 3q has been most frequently reported in HSIL and VSCC (Lin et al., 1998; Flowers et al., 1999; Pinto et al., 1999; Rosenthal et al., 2001; Allen et al., 2002; Bryndorf et al., 2004; Osakabe et al., 2007; Yangling et al., 2007; Aulmann et al., 2008). LOH at chr 2.4 and chr 8.2 has been identified in both HPV-related VSCC, and the adjacent HSIL (Lin et al., 1998). Single studies have reported loss of chr 13q, and gain of chr 20p and chr 20q for both HSIL and VSCC (Flowers et al., 1999; Bryndorf et al., 2004). In addition, gain of chr 8q, and loss of chr 8p, chr 11, and chr 17 in VSCC have been reported (Flowers et al., 1999; Pinto et al., 1999; Rosenthal et al., 2001; Allen et al., 2002; Bryndorf et al., 2004;

Yangling et al., 2007).

For HSIL that progressed to VSCC, Swarts et al. identified chr 1p, 1q, 3q, 20 gains and chr 4 loss (Swarts et al., 2018). Interestingly, in VSCC samples with an adjacent HSIL, chr 1pq gains could be detected only in the HSIL, and not in the VSCC, potentially implying intratumoral heterogeneity (Swarts et al., 2018). Swarts et al. also observed distinct patterns of CNA in HSIL and dVIN, and that CNA were more frequent in HSIL than in dVIN (Swarts et al., 2018).

3.2.5.2. Somatic mutations. Nooij et al. detected *TP53* mutations in both HSIL and VSCC, albeit in much lower frequency than in HPV-independent counterparts (Nooij et al., 2017). Contrasting findings were reported by Zięba et al., who detected *TP53* mutations with comparable frequency in HPV-related, and HPV-independent VSCC (Zięba et al., 2018). They ascribed this difference to sequencing techniques and the highly sensitive HPV-detection method used (Zięba et al., 2018).

HRAS mutations have been detected in both HSIL and VSCC (0–14%), whereas *NOTCH1* mutations have been detected only in HSIL (Nooij et al., 2017; Weberpals et al., 2017). Other mutations that have been detected in HPV-related VSCC include *PIK3CA* (7–33%), *CDKN2A* (0–25%), *PTEN2* (9 %), *FGFR3* (14 %), *KIT* (18 %), *BRCA2* (17 %), *FBXW7* (3–17%) (Nooij et al., 2017; Zięba et al., 2018; Weberpals et al., 2017; Han et al., 2018).

3.2.5.3. Microsatellite instability (MSI). Only a single study has reported MSI for HPV-related VSCC (Lin et al., 1998).

3.2.5.4. Epigenetic changes. Hypermethylation of *CDKN2A* (9.1–15.4%), *Stratifin* (45.5–53.8%), *TSR1* (20 %), and *TSLC1* (44.4 %), has been reported for HSIL and VSCC, using methylation-specific PCR (ms-PCR) (Gasco et al., 2002; Guerrero et al., 2011; O'Nions et al., 2001).

3.2.5.5. Expression profiling. Upregulation of genes involved in cell-cycle regulation and proliferation, with the exception of cyclins D1 and D2, has been reported for HSIL (Santegoets et al., 2007). Cyclins D1 and D2 were significantly downregulated, probably due to the pronounced upregulation of *CDKN2A* (Santegoets et al., 2007). Upregulation of Fanconi genes (*FANCA*, *FANCD2,* and *FANCE)*, and *BRCA1* have been reported in HSIL, which probably reflects the response to the HPV-induced DNA damage. Downregulation of nuclear androgen and estrogen receptors (AR and ESR1) potentially indicates reduced paracrine and endocrine regulation (Santegoets et al., 2007). Cell-cell adhesion molecules (*ASAM, SLIT2, ITGA8, FN1, EPDR1*) were also downregulated in HSIL, indicating its tendency towards invasion (Santegoets et al., 2007, 2012).

3.2.6. GEO DataSet analysis

Based on the expression levels of p16 (*CDKN2A*), 3 samples were identified as HPV-related VSCC. In these samples, expression of 1117 probe sets (675 up and 442 down) differed significantly from the controls. Of these, 342 probe sets (244 up and 98 down) were found to be exclusively involved in HPV-related VSCC, i.e. not involved in HPV-independent VSCC. The associated biological processes, canonical pathways, and the upstream regulators of these differentially expressed genes are discussed below.

3.2.6.1. Biological processes. The associated biological processes were identified through Gene Ontology (GO) analysis using DAVID. Those with the most significant p-values are depicted in Fig. 4. These included processes related to host immune response to HPV-infection, organismal injury and death, and proteolytic activity mediated by proteasomes through ubiquitin-ligation.

3.2.6.2. Pathways. Based on the –log(p-values) of the differentially expressed genes, the associated canonical pathways were identified using IPA, and are depicted in Fig. 5. The protein ubiquitination pathway was most significantly upregulated. This signaling pathway helps degrade regulatory proteins involved in cell proliferation, apoptosis, DNA repair, and antigen presentation. Several cancers are known to manipulate the ubiquitin pathways to ensure tumor cell survival and metastasis. Other significantly upregulated pathways i.e. antigen presentation pathway, interferon signaling, and phagosome maturation, regulate cellular immune response. The systemic lupus erythematosus (SLE)-signaling pathway mediates aberrant activation of T-cells, and is known to play a role in cellular growth and proliferation.

3.2.6.3. Upstream regulators. Based on the z-scores, 95 upstream regulators (74 activated, 21 inhibited) were identified using IPA [Table S2, supplementary document 2]. These comprised protein complexes, cytokines, enzymes, G-protein coupled receptors, transmembrane receptors, growth factors, ligand-dependent nuclear receptors, miRNAs, transcription and translation regulators, and transporters. The top five activated upstream regulators were the cytokines, tumor necrosis factor (TNF), interferons gamma and alpha2 (IFNG, IFNA2) and prolactin (PRL), and the transcription regulator, interferon regulatory factor 7 (IRF7). All of these are primarily associated with pro-inflammatory functions, and host-response to viral infections.

3.3. HPV-independent VSCC precursors (dVIN)

3.3.1. Etiopathogenesis

HPV-independent VSCC has been associated with lichen sclerosus (LS), and occasionally with LSC, although the exact mechanism of this transformation remains unclear. The 'scar cancer' model has been proposed as a possible explanation, where chronic inflammation leads to repeated epithelial injury and incites malignant transformation (Cohen et al., 2019).

Women with LS have a reported relative risk of 38.4 for the development of dVIN and VSCC (Bigby et al., 2016). Long-standing LS, and low compliance with high-dose potent topical steroid ointments have been associated with higher rates of (pre)malignant transformation (Bigby et al., 2016).

The immune-microenvironment of LS resembles that of an autoimmune disorder, characterized by a high concentration of CD8+, and FOXP3+ T-cells, and T-cell receptor rearrangements; this probably provides a fertile ground for carcinogenesis (Wenzel et al., 2007; Terlou et al., 2012; Regauer et al., 2002).

TP53 mutations (missense, deletions, and nonsense) have been identified in both dVIN and VSCC, and have therefore been implicated in their pathogenesis (Hoang et al., 2016; Regauer et al., 2019). However, recent studies indicate that not all HPV-independent VSCC follow the *TP53* pathway, this will be discussed later.

3.3.2. Clinical features

Although more common amongst post-menopausal women of 60–80 years, dVIN has also been reported in women < 40 years of age (Yang and Hart, 2020). Clinically, dVIN often produces unifocal grey-white discolorations with a roughened surface, and less frequently whitish plaques, or nodules (Yang and Hart, 2020). The clitoris is frequently involved. Perineal involvement is less common than in HSIL (Hinten et al., 2018; McAlpine et al., 2017). Itching and burning sensations are common presentations, but occasionally, dVIN can be asymptomatic (Hoang et al., 2016).

dVIN is less frequently diagnosed as a stand-alone lesion, than adjacent to VSCC. This has been attributed to under-diagnosis of dVIN, due to its subtle histological appearance, and rapid progression to VSCC (Eva et al., 2009). The median interval between biopsy of dVIN and diagnosis of VSCC has been reported to be 43.5 months (range 8–102 months) (Bigby et al., 2016). VSCC arising in the background of dVIN has poorer prognosis (overall survival and disease specific survival), and recurs more commonly than VSCC associated with HSIL (Hinten

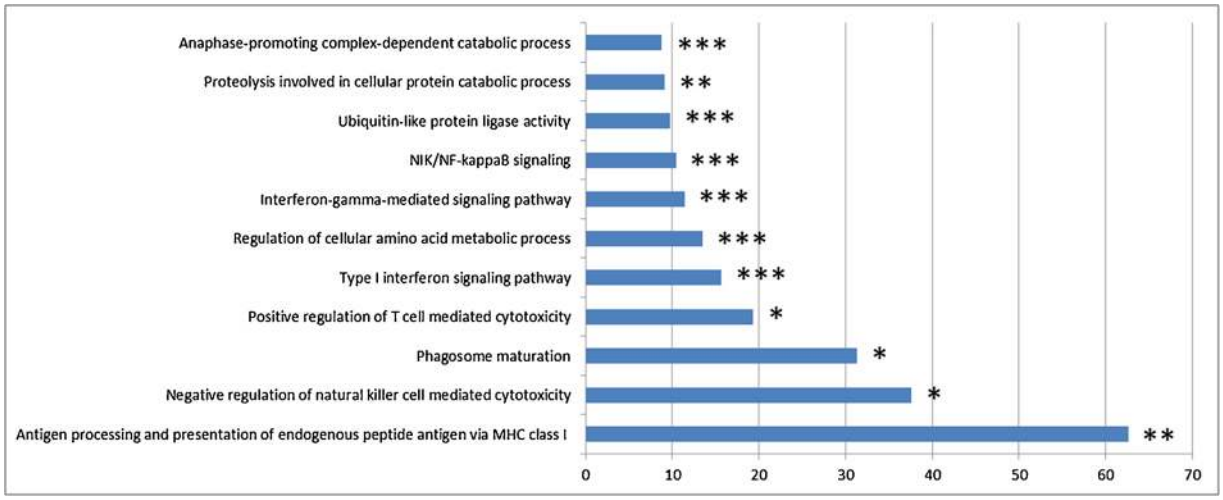

Fig. 4. Biological processes involved in HPV-related vulvar squamous cell carcinoma (VSCC).
DAVID GO enrichment analysis (biological process) of differentially expressed genes in HPV-related VSCC. The figure shows the most significant biological processes ($p < 0.01$) on the y-axis, and the fold enrichment on the x-axis. Asterisks indicate the p-values, *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$

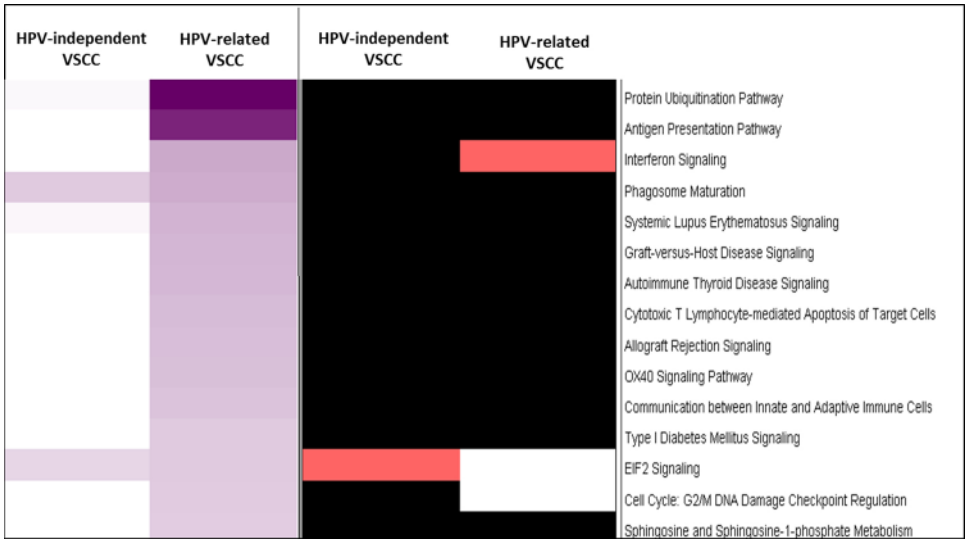

Fig. 5. Canonical pathways involved in HPV-related vulvar squamous cell carcinoma (VSCC).
Left: The top 15 canonical pathways regulated with statistical significance in HPV-related VSCC are shown, along with comparative regulation of these pathways in HPV-independent VSCC. Color-coding of the map corresponds to the -log(p-value) of each canonical pathway, calculated by Fisher's exact test, right-tailed.
Right: Color-coding corresponds to the z-scores; red indicates predicted pathway activation, and black indicates that no predictions were available

et al., 2018; Eva et al., 2009). A history of prior, synchronous, or subsequent VSCC can be present in upto 85.7 % of dVIN (Bigby et al., 2016).

3.3.3. Histological features

Histological features of dVIN were extracted from 11 studies (Preti et al., 2014; Hart, 2001; Hoang et al., 2016; Cohen et al., 2019; Yang and Hart, 2020; Bigby et al., 2016; Reutter et al., 2016; van den Einden et al., 2013; Dasgupta et al., 2018; Singh et al., 2015; Ordi et al., 2009). The 'differentiated' appearance of dVIN results from premature keratinization in the deeper epithelial layers, which is a consequence of disturbed maturation [Fig. 6].. This overtly eosinophilic appearance due to premature keratinization can be readily identified even under low magnification (Hoang et al., 2016; Cohen et al., 2019). Unlike HSIL, nuclear atypia in dVIN is often limited to the basal layers (Preti et al., 2014; Hart, 2001; Yang and Hart, 2020).

The histological diagnosis of dVIN suffers from poor reproducibility (van den Einden et al., 2013). A panel of experts from ISSVD consensually accepted basal layer atypia as the only essential diagnostic feature. Histological features arbitrated as 'strongly supportive' included basal layer hyperchromasia, basal layer mitoses, and large keratinocytes with abundant eosinophilic cytoplasm (Reutter et al., 2016). Features of disturbed maturation, such as individual cell keratinization, presence of squamous whorls and keratin pearls just above the basal layer, and cobblestoning have been reported to be particularly helpful for dVIN cases where nuclear atypia is difficult to discern (van den Einden et al., 2013; Dasgupta et al., 2018). The histological features are listed in Table 1.

dVIN can sometimes be hard to distinguish from the reactive changes seen in LS, or LSC. Singh et al. therefore recommended that biopsies should include the interface between the lesion and normal skin, as this allows appreciation of the abrupt edges of dVIN, which lichenoid lesions lack (Singh et al., 2015).

Infrequently, dVIN exhibits full thickness moderate to severe atypia, similar to HSIL. These lesions are referred to as HSIL-like dVIN, or dVIN-with basaloid morphology, [] and can be distinguished from HSIL by the lack of koilocytes, and in certain cases, by the presence of focal conventional dVIN-like areas (Ordi et al., 2009).

3.3.4. Immunohistochemical markers

Immunohistochemical markers studied in dVIN are presented below, and summarized in Table 2. This information was extracted from

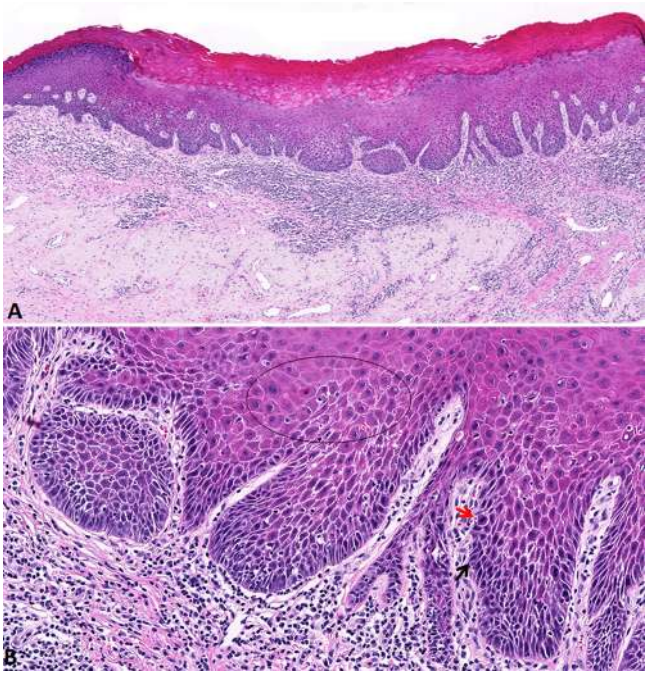

Fig. 6. Differentiated vulvar intraepithelial neoplasia with characteristic histological features; hematoxylin-eosin (HE) stain. A. A widened epithelium with hyperkeratosis, parakeratosis, and an eosinophilic appearance can be appreciated under low magnification (original magnification 25X). B. Nuclear atypia, particularly prominent in the basal layers, can be appreciated under higher magnification (original magnification 200X). Angulated nuclei (black arrow) and mitotic figure (red arrow) are observed in the basal layer. Cobblestone appearance, individual cell keratinisation, and macronucleoli are present (circled area) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

20 studies (van der Avoort et al., 2006; Yang and Hart, 2020; Hoevenaars et al., 2008; Rolfe et al., 2001; Brustmann and Brunner, 2013; Goyal et al., 2018; Wellenhofer and Brustmann, 2012; Pinto et al., 2013; Stewart and Crook, 2014; Podoll et al., 2016; Dasgupta et al., 2018; Singh et al., 2015; Ordi et al., 2009; Santos et al., 2004; Hantschmann et al., 2005; Liegl and Regauer, 2006; Rolfe et al., 2003; Vanin et al., 2002; Jin and Liang, 2019; Zannoni et al., 2011). The immunohistochemical markers have been categorized as per their subcellular location as nuclear, or cytoplasmic. The biological processes and canonical pathways associated with these markers are presented in Table S1 (supplementary document 2).

3.3.4.1. Nuclear markers

(i) p53: The underlying *TP53* mutations in dVIN can be reflected as over-expression/mutation pattern, or null-pattern on p53-IHC (Yang and Hart, 2000; Santos et al., 2004; Hantschmann et al., 2005). In mutation pattern, p53-staining in > 90 % cells of the basal layer, along with suprabasal extension, ranging from lower one-third to full epithelial thickness is seen, and this is associated with missense mutations [Fig. 7] (Santos et al., 2004; Hantschmann et al., 2005). In null pattern on the other hand, complete negativity is noted. This is seen in up to 27 % cases of dVIN, and is associated with nonsense mutations (Singh et al., 2015).

A proportion of dVIN exhibits wild type (wt) p53-staining pattern (Nooij et al., 2017). Further delineation of the histological and molecular characteristics of these lesions is needed to determine whether they represent a subset of dVIN, or a distinct category of precursor of HPV-independent VSCC.

p53-IHC however, has limitations in distinguishing dVIN from lichenoid conditions. Increased p53-staining, named as 'p53-wt overexpression' can be seen in 5–61% cases of LS, and up to 40 % cases of squamous hyperplasia, due to oxidative stress (Santos et al., 2004; Hantschmann et al., 2005; Liegl and Regauer, 2006; Rolfe et al., 2003; Vanin et al., 2002). Moreover, p53-positivity has also been noted in 'normal'-appearing vulvar skin, similar to the p53-signatures in the fallopian tube and endometrium, the significance of which is yet unknown (Jin and Liang, 2019).

(ii) p16: Complete negativity, to minimal focal staining with p16-IHC has been reported for dVIN [Fig. 7] (Hoevenaars et al., 2008). For dVIN histologically mimicking HSIL (HSIL-like dVIN), negative p16-IHC, and mutation-pattern or null-pattern expression with p53-IHC [Fig. 7] helps distinguish it from HSIL (Ordi et al., 2009).
(iii) Cyclin-D1: Nuclear cyclin-D1-expression has been reported to be mildly increased in dVIN, compared to normal vulvar epithelium. However, an almost identical staining pattern is seen in reactive vulvar disorders (Rolfe et al., 2001).
(iv) SOX2: Increased, strong SOX2-staining in the nuclei of basal and parabasal keratinocytes is seen in dVIN. In LS, only weak and occasional SOX2-staining from the basal to the upper epithelial layers is seen. SOX2-staining patterns in dVIN and LS have been reported to differ significantly ($p < 0.0001$) (Brustmann and Brunner, 2013).
(v) GATA3: Partial to complete loss of nuclear expression of GATA3 in the basal layer, with or without loss in the parabasal layer has been reported for dVIN (Goyal et al., 2018). Since strong GATA3-staining has been noted in LS and LSC, GATA3 has been proposed as a potential diagnostic adjunct for dVIN (Goyal et al., 2018).
(vi) hTERT: Nuclear staining with hTERT in dVIN has been reported to follow the distribution of the atypical cells in the epithelium, and to be significantly higher than that in LS (Wellenhofer and Brustmann, 2012).
(vii) Estrogen and progesterone receptors (ERα, ERß, and PR): In dVIN adjacent to VSCC in elderly patients, no nuclear expression of ERα was seen, whereas in LS, ERα-expression in the middle and lower-thirds of the epithelium was noted. For ERß, nuclear/cytoplasmic expression has been reported in both dVIN and LS. Scant, nuclear PR-expression has been reported in normal vulvar epithelium, LS, and dVIN (Zannoni et al., 2011).
(viii) Ki-67/MIB-1: Increased MIB-1-expression is seen in the basal and parabasal layers in dVIN. This can be helpful in distinguishing dVIN from LS, which usually shows only basal MIB-1-expression (van der Avoort et al., 2006; Hoevenaars et al., 2008).
(ix) p-S6: Increased nuclear p-S6-expression in the basal/parabasal layers, or extending across full epithelial thickness has been reported for dVIN. In contrast, p-S6-expression in LS was reported to be limited to the basal layers (Pinto et al., 2013).

3.3.4.2. Cytoplasmic markers

(i) FSCN1: Diffuse cytoplasmic FSCN1-staining has been reported for dVIN, with some cases showing concurrent nuclear staining (Stewart and Crook, 2014).
(ii) CK17: Intermediate to strong, diffuse, cytoplasmic CK17-expression, in the suprabasal layers to full epithelial thickness has been reported in dVIN (Podoll et al., 2016). CK17-expression has been reported to be significantly higher in dVIN compared to LS (Dasgupta et al., 2018).
(iii) CK13: Weak cytoplasmic CK13-expression in the suprabasal or superficial epithelial layers, or complete loss of CK13-expression have been reported for dVIN (Dasgupta et al., 2018). For LS, patchy/diffuse CK13-expression of moderate intensity, in the suprabasal layers of the epithelium has been reported (Dasgupta et al., 2018).

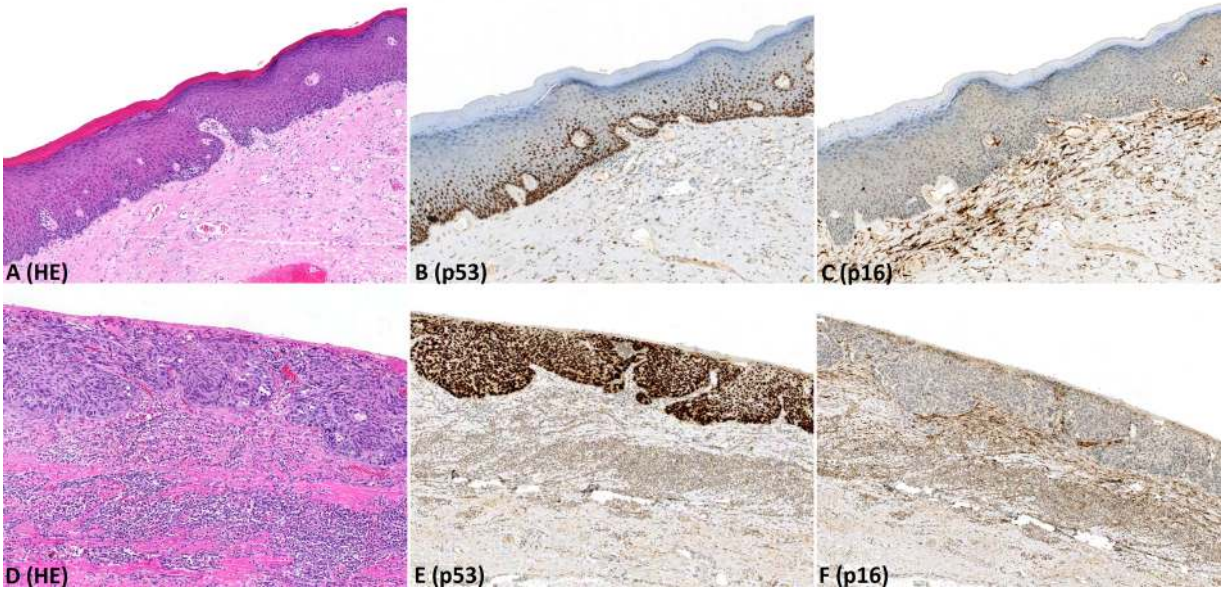

Fig. 7. Differentiated vulvar intraepithelial neoplasia (dVIN), histology and p53 and p16 immunohistochemistry (IHC), original magnification 50X.
A-C: dVIN with typical histology
A. Hematoxylin-Eosin (HE) stained section shows the characteristic histology of dVIN
B. On p53-IHC, mutation pattern staining, i.e. positive staining in the basal epithelial layers extending into the upper one-third of the epithelium is seen
C. Complete negativity is noted with p16-IHC
D-F: HSIL-like dVIN
D. HE stained section shows dVIN with full epithelial thickness atypia, which is more commonly noted in HSIL
E. Mutation pattern staining is noted with p53-IHC
F. Complete negativity is noted with p16-IHC

3.3.5. Molecular markers

A Allelic imbalances, LOH, CNA: Loss of chr 2.4, 5.2, 3p, 13q, and 17p has been detected in both dVIN and HPV-independent VSCC (Lin et al., 1998; Flowers et al., 1999). Loss of 3p and 17p has been most frequently reported (Flowers et al., 1999; Pinto et al., 1999; Rosenthal et al., 2001; Osakabe et al., 2007; Yangling et al., 2007; Rolfe et al., 2003). Alterations in chr 8p23.1, 8p23.3, and 8p11.22, and chr 3 and chr 8 isochromosome formation (3p/8p loss with 3q/8q gain) have been more commonly detected in HPV-independent VSCC than in HPV-related VSCC (Swarts et al., 2018). Gains in chr 8q, and losses in chr 1p, 3p, and 8p have been reported to be more frequent in dVIN, than in HSIL (Swarts et al., 2018).

B Somatic mutations: Mutations of *TP53* are the most frequent somatic mutations in dVIN and HPV-independent VSCC (Brooks et al., 2000; Trietsch et al., 2015). Frequency of *TP53* mutations in VSCC, in studies using next generation sequencing (NGS) or whole exome sequencing (WES), varied between 41–79% (Nooij et al., 2017; Zięba et al., 2018; Weberpals et al., 2017; Han et al., 2018; Watkins et al., 2017). Other somatic mutations that have been detected in VSCC, albeit in lower frequency, include *HRAS* (3–31%), *NOTCH1* (28–41%), *FGRF3* (4.8 %), *CDKN2A* (11–36%), *FBXW7* (0–11%), *PIK3CA* (0–19%), *PPP2R1A* (3 %), and *EGFR* (Cohen et al., 2019; Nooij et al., 2017; Zięba et al., 2018; Weberpals et al., 2017; Han et al., 2018). In lesions diagnosed histologically as dVIN, and showing wt-p53 expression on IHC, Nooij et al. detected mutations in *NOTCH1* (20 % of cases) and *HRAS* (10 % of cases) (Nooij et al., 2017).

C MSI: Bujko et al. could not detect MSI in HPV-independent VSCC, while Pinto et al. detected MSI in 27 % of dVIN and 12 % of LS cases in his study (Bujko et al., 2012; Pinto et al., 2000).

D Epigenetic changes: Hypermethylation of *CDKN2A*, *Stratifin*, *RASSF1A*, *RASSF2A*, *TSP-1*, and *MGMT* have been reported for both dVIN and VSCC on ms-PCR, with a higher frequency in VSCC, than in dVIN (Gasco et al., 2002; Guerrero et al., 2011; Soufir et al., 2007).

3.3.6. GEO DataSet analysis

Three samples from the included dataset were identified as HPV-independent VSCC, in which the expression of 1158 probe sets (545 up and 613 down) significantly differed from the control. Of these, 382 probe sets (203 up and 179 down) were exclusive for HPV-independent VSCC. The associated biological processes, canonical pathways, and upstream regulators were identified using DAVID, and IPA, and are discussed below.

3.3.6.1. Biological processes. The upregulated biological processes with the most significant p-values are depicted in Fig. 8. These included biological processes associated with the transcription factor CREB, metabolic processes, and reduced response to hormonal stimulation, and cellular senescence.

3.3.6.2. Pathways. Based on the –log(p-values) of the differentially expressed genes, the associated canonical pathways were identified, and depicted in Fig. 9. Metabolic pathways known to play a role in cell morphology, and embryonic development were most significantly upregulated. This is probably a reflection of the metabolic re-programming exhibited by solid tumors, to sustain proliferation and survival. Aryl hydrocarbon receptor signaling was the only signaling pathway in the top 5, which is known to down-regulate TGF-ß mediated apoptosis.

3.3.6.3. Upstream regulators. Based on the z-scores, 57 upstream regulators (13 activated, 44 inhibited) were identified [Table S3, supplementary document 2]. Similarly to HPV-related VSCC, these comprised protein complexes, cytokines, enzymes, growth factors, G-protein coupled receptors, mature miRNAs, ligand-dependent nuclear receptors, transcription and translation regulators, and transporters.

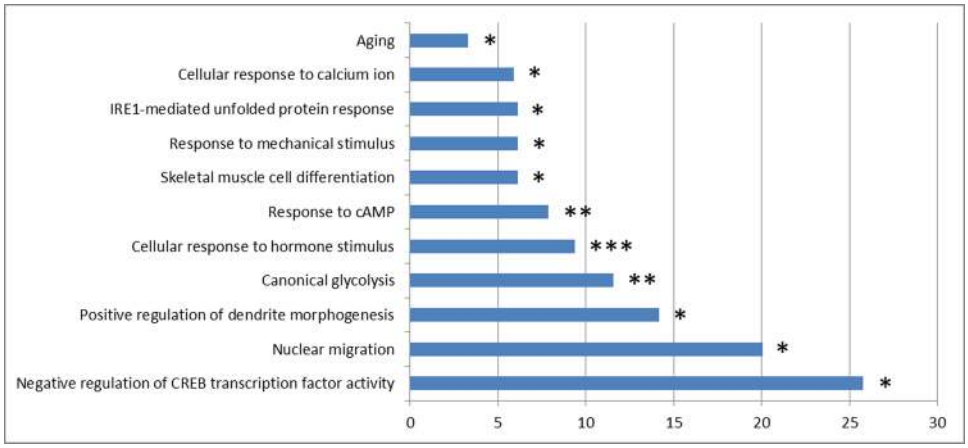

Fig. 8. Biological processes involved in HPV-independent vulvar squamous cell carcinoma (VSCC).
DAVID GO enrichment analysis (biological process) of differentially expressed genes in HPV-independent VSCC. The figure shows the most significant biological processes (p-value < 0.01) on the y-axis, and fold enrichment on the x-axis. Asterisks indicate the p-values*, $p < 0.01$; **$p < 0.001$; ***$p < 0.0001$

The top five activated upstream regulators included 4 transcription regulators i.e. MYCN proto-oncogene, NK2 homeobox 3 (NKX2-3), aryl hydrocarbon receptor nuclear translocator (ARNT), and SOX2, and 1 mature microRNA (miR-125b-5p). MYCN, ARNT, and miR-125b-5p are known to regulate *TP53*, while SOX2 is regulated by *TP53*.

3.4. HPV-independent VSCC precursors (others)

Atypical verruciform lesions, with histological appearance distinct from dVIN, are other putative precursors of HPV-independent VSCC. Their etiopathogenesis, rates of malignant transformation, and molecular profiles remain largely unknown.

An example from this group is vulvar acanthosis with altered differentiation (VAAD) (Nascimento et al., 2004). The typical histological features of VAAD are acanthosis with variable verruciform architecture, loss of granular layer with superficial epithelial pallor, and multilayered plaque-like parakeratosis (Nascimento et al., 2004).

Watkins et al. recently described a category of atypical verruciform lesions, bearing *PIK3CA* and *ARID2* mutations, instead of *TP53* mutations. These lesions, with an exophytic, acanthotic, or verruciform architecture, and lacking the typical features of HSIL, or sufficient basal atypia for a diagnosis of dVIN, were named 'differentiated exophytic vulvar intraepithelial lesion (DEVIL)' (Watkins et al., 2017).

4. Discussion

Concepts of VSCC precursors have significantly evolved since the first description in 1912, and a summary of current knowledge, with emphasis on histology and biomarkers, is presented in this review. As we included only English language literature, and articles with full-text availability, some relevant information may have been missed. The limited number of studies on immunohistochemical and molecular markers of VSCC precursors, and the small sample sizes in most of these, did not permit a meta-analysis. These are potential limitations of this review. Nevertheless, we discuss the important insights into VSCC and its precursors gained from the literature.

The WHO, ISSVD, and LAST committees have made commendable efforts to introduce and advocate standardized terminology for VSCC precursors. This allows better transfer of knowledge between clinicians, pathologists, and patients, and also ensures reproducibility in research. However, terminologies need modification in the face of newer information.

For dVIN, it is apparent that (i) malignant transformation is more frequent than HSIL, and (ii) occasionally it exhibits the histology typically associated with HSIL. In view of these 'high-grade' features, the legitimacy of the attribute 'differentiated' might be questioned. The merit of using HPV-independent/HPV-negative HSIL/high-grade VIN, in place of dVIN, may be deliberated.

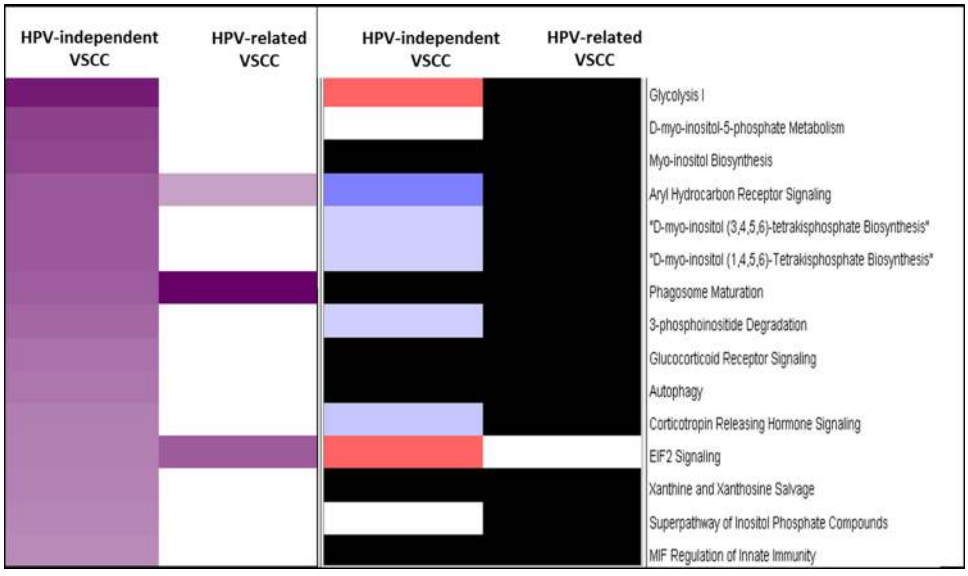

Fig. 9. Canonical pathways involved in HPV-independent vulvar squamous cell carcinoma (VSCC).
Left: The top 15 canonical pathways regulated with statistical significance in HPV-independent VSCC are shown, along with comparative regulation of these pathways in HPV-related VSCC. Color-coding of the map corresponds to the -log(p-value) of each canonical pathway, calculated by Fisher's exact test, right-tailed.
Right: Color-coding corresponds to the z-scores; red indicates predicted pathway activation, blue indicates predicted inhibition, and black indicates that no predictions were available

Since dVIN is known to develop in patients with chronic vulvar dermatoses, biopsies should be performed for lesions recalcitrant to therapy, or with a suspicious clinical appearance, to rule out dVIN. Adequate sampling is also of utmost importance in these cases, as VSCC is often diagnosed adjacent to dVIN (Preti et al., 2014).

Regarding HPV-independent VSCC and dVIN, several facets of the pathogenesis still remain unknown. The association of LS and dVIN is widely reported in the literature, but a history of LS is not universally present in women with dVIN. Reports of p53-wt dVIN, and other putative precursors, e.g. DEVIL, indicate that HPV-independent VSCC and its precursors constitute a heterogeneous category, with potentially different pathogenesis and natural history.

The clinical, histological, and molecular characteristics of the newly identified putative precursors need better delineation. Routine clinical photographs of vulvar lesions suspected to be premalignant may be useful in this context. One of the histological diagnostic criteria for DEVIL, i.e. 'insufficient basal atypia to warrant a diagnosis of dVIN', can be considered highly subjective. Furthermore, the malignant potential of these lesions needs to be established through prospective studies.

As an ancillary tool for the histological diagnosis of dVIN and HSIL, a reasonably wide assortment of immunohistochemical markers has been evaluated. Nevertheless, p53 and p16-IHC remain the most widely used in the clinical setting. Most studies on immunohistochemical markers included small numbers, and rarely did more than one study assess the same immunohistochemical marker. This has deterred their translation to the clinics.

Since p53 has limited value in discriminating dVIN from lichenoid lesions, immunohistochemical markers with higher specificity for dVIN, especially based on genetic and epigenetic profile, need to be explored. In view of the existence of cases of dVIN and HSIL with overlapping histology, routine use of p16-IHC to aid their accurate categorization should be considered, as the treatment and prognosis of both lesions differ significantly. Routine use of p16-IHC, as a surrogate marker of HPV-status, should also be considered for VSCC, as several studies have demonstrated poorer survival and higher recurrence rates for HPV-independent VSCC.

Studies on molecular characterization of VIN are limited, but they provide a few key findings. Intratumoral heterogeneity has been identified in HPV-related VSCC. For HPV-independent VSCC and precursors, genotypic subtypes (p53-wt category) with distinct mutational profiles have been identified. These data require validation in independent, larger cohorts, using whole genome sequencing. Epigenetic alterations, and the role of MSI are underexplored areas in VSCC, and may provide useful information for diagnosis, or prognostication.

Our results from GEO analyses demonstrate the differences in pathways involved in HPV-related, and HPV-independent VSCC. Canonical pathways related to host-pathogen interactions were exclusively upregulated in HPV-related VSCC, whereas metabolic pathways influenced by *TP53* played a more significant role in HPV-independent VSCC. The categories of upstream regulators also differed; pro-inflammatory cytokines were involved in HPV-related VSCC, whereas transcription regulators related to *TP53* were more operative in HPV-independent VSCC. A limitation of our results is the small sample size of the GEO dataset that was analyzed. Whether these processes and pathways are similarly involved in the precursors of the corresponding VSCC categories deserves exploration, as this may allow identification of potential therapeutic targets to improve clinical management.

5. Conclusion

The category of HPV-independent VSCC and its precursors needs better histological, immunohistochemical, and molecular delineation. Combination of advanced sequencing techniques, and leverage of bioinformatics can pave the path for molecular characterization of vulvar (pre)malignancies, and personalized treatment.

Declaration of competing Interest

The authors have no competing interests to declare. No external funding was received for this study. All the authors gave their approval for the version to be published and agree to be accountable for all aspects of the study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.critrevonc.2020.102866.

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